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The *Agrobacterium tumefaciens atu3184* gene, a member of the COG0523 family of GTPases, is regulated by the transcriptional repressor Zur

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ABSTRACT

Analysis of the *Agrobacterium tumefaciens* C58 genome revealed a potential Zur (zinc uptake regulator) binding site (5′-GATATGTTATTACATTAC-3′, the underlined letters are the center of symmetry of the inverted palindrome) located in the upstream region of *atu3184,* whose gene product is a member of the COG0523 subfamily of G3E GTPases. The specific interaction of the Zur protein with the 18-bp inverted repeat operator motif in the presence of zinc was demonstrated *in vitro* by a DNA band shift assay and a DNase I footprinting assay. A LacZ reporter fusion assay further confirmed that Zur negatively regulates *atu3184* promoter activity *in vivo*. The expression of *atu3184* was upregulated in response to zinc limitation in the wild-type strain, but the *zur* mutant strain exhibited high-level constitutive expression of *atu3184* under all conditions, irrespective of the zinc levels. It is likely that *A. tumefaciens* Zur senses zinc and directly regulates the *atu3184* promoter by a molecular mechanism similar to that of *Escherichia coli* Zur, where the operator DNA is surrounded by four Zur monomers forming two dimers bound on the opposite sides of the DNA duplex. Disruption of *atu3184* did not affect cell growth under metal-limited conditions and had no effect on the total cellular zinc content. Furthermore, an *A. tumefaciens* strain lacking *atu3184* caused a tumor disease in a host plant.

1. Introduction

GTPases can bind and hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP) and inorganic phosphate. Regulatory GTPases are known as molecular switches because they alternate between an inactive GDP-bound state and an active GTP-bound state that can modulate a variety of cellular processes (Wittinghofer and Vetter, 2011). COG0523 proteins belong to the G3E family of P-loop GTPases (Haas et al., 2009). G3E family proteins function as metal insertases and/or as metallochaperones. The insertase function facilitates metal incorporation into active sites of target metalloproteins, whereas the metallochaperone function is involved in the storage and delivery of metals to target metal-requiring proteins (Haas et al., 2009). The first member of the COG0523 family, named *cobW*, was identified in *Pseudomonas denitrificans* (Crouzet et al., 1991) and is located in a cluster of genes involved in cobalamin (vitamin B_{12}) biosynthesis. Although it has not yet been experimentally verified, *P. denitrificans* CobW is believed to deliver cobalt to the cobaltochelatase complex (CobNST) during the process of cobalt insertion into the corrin ring (Heldt et al., 2005). In addition to the CobW subgroup, the COG0523 family members can be separated further into at least fifteen subgroups. Zur (zinc uptake regulator)-regulated COG0523, due to the COG0523-encoding genes downstream from putative Zur binding sites, is a large subgroup in which function is particularly linked to zinc homeostasis (Haas et al., 2009). The Zur-regulated COG0523 subgroup includes YciC from *Bacillus subtilis* ($YciC_{Bs}$) (Gaballa and Helmann, 1998), Zrg from *Corynebacterium diphtheriae* (Zrg_{Cd}) (Smith et al., 2009), ZigA from *Acinetobacter baumannii* ($ZigA_{Ab}$) (Mortensen et al., 2014), YciC from *Agrobacterium tumefaciens* ($YciC_{At}$) (Chaoprasid et al., 2016) and CobW1 from *Cupriavidus metallidurans* ($CobW1_{Cm}$) (Bütof et al., 2017). The COG0523 proteins YeiR (Blaby-Haas et al., 2012) and YjiA (Khil et al., 2004; Sydor et al., 2013) from *Escherichia coli* ($YeiR_{Ec}$ and $YjiA_{Ec}$, respectively) have also been shown to be involved in zinc homeostasis, although there is no Zur binding site in their promoter regions; thus, the

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Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or characteristics	Reference or source
***A. tumefaciens* strains**		
NTL4	Wild-type (WT) strain, a Ti plasmid-cured derivative of strain C58	Luo et al. (2001)
PC135	*zinT*::pKNOCK-Gm (*zinT*::Gm), Gm^r	Bhubhanil et al. (2014c)
PS132	*znuA*::pKNOCK-Gm (*znuA*::Gm), Gm^r	Bhubhanil et al. (2014c)
SPP12	*zur*::pKNOCK-Gm (*zur*::Gm), Gm^r	Bhubhanil et al. (2014c)
TC142	*troC*::pKNOCK-Gm (*troC*::Gm), Gm^r	Chaoprasid et al. (2016)
YC154	*yciC*::pKNOCK-Km (*yciC*::Km), Km^r	Chaoprasid et al. (2016)
TCYC15	*troC*::pKNOCK-Gm and *yciC*::pKNOCK-Km (*troC*::Gm *yciC*::Km), Gm^r and Km^r	Chaoprasid et al. (2016)
ZTYC15	*zinT*::pKNOCK-Gm and *yciC*::pKNOCK-Km (*zinT*::Gm *yciC*::Km), Gm^r and Km^r	Chaoprasid et al. (2016)
ZG3184	*atu3184*::pKNOCK-Gm (*atu3184*::Gm), Gm^r	This study
ZG3184TC	*atu3184*::pKNOCK-Gm and *troC*::pKNOCK-Km (*atu3184*::Gm *troC*::Km), Gm^r and Km^r	This study
ZG3184YC	*atu3184*::pKNOCK-Gm and *yciC*::pKNOCK-Km (*atu3184*::Gm *yciC*::Km), Gm^r and Km^r	This study
ZG3184ZA	*atu3184*::pKNOCK-Gm and *znuA*::pKNOCK-Km (*atu3184*::Gm *znuA*::Km), Gm^r and Km^r	This study
ZG3184ZT	*atu3184*::pKNOCK-Gm and *zinT*::pKNOCK-Km (*atu3184*::Gm *zinT*::Km), Gm^r and Km^r	This study
***E. coli* strains**		
BW20767	Host for plasmids pKNOCK-Gm and pKNOCK-Km	Metcalf et al. (1996)
DH5α	Host for general DNA cloning	Grant et al. (1990)
Plasmids for gene inactivation		
pKNOCK-Gm	Suicide vector, Gm^r	Alexeyev (1999)
pKNOCK3184	Internal coding region of *atu3184* cloned into pKNOCK-Gm, Gm^r	This study
pKNOCKmZINT	Internal coding region of *zinT* cloned into pKNOCK-Km, Km^r	Chaoprasid et al. (2016)
pKNOCKmYCIC	Internal coding region of *yciC* cloned into pKNOCK-Km, Km^r	Chaoprasid et al. (2016)
pKNOCKmZNUA	Internal coding region of *znuA* cloned into pKNOCK-Km, Km^r	Chaoprasid et al. (2016)
pKNOCKmTROC	Internal coding region of *troC* cloned into pKNOCK-Km, Km^r	This study
Plasmids for complementation		
pBBR1MCS-2	Expression vector, Km^r (pBBR)	Kovach et al. (1995)
pBBR1MCS-4	Expression vector, Ap^r	Kovach et al. (1995)
pZUR	Full-length *zur* cloned into pBBR1MCS-2, Km^r	This study
pATU3184	Full-length *atu3184* cloned into pBBR1MCS-4, Ap^r	This study
pYCIC	Full-length *yciC* cloned into pBBR1MCS-4, Ap^r	Chaoprasid et al. (2016)
Plasmids for protein expression and purification		
pASK-IBA3	Protein expression vector, Ap^r	IBA
pZur-Step-tag	Coding region of *zur* cloned into pASK-IBA3, Ap^r	This study
Plasmids for EMSA		
pBluescript II KS	Cloning vector	Stratagene
pPAtu3184	283 bp *atu3184* promoter region containing native Zur box cloned into pBluescript II KS, Ap^r	This study
pP#Atu3184	A derivative of pPAtu3184 containing mutated Zur box, Ap^r	This study
Plasmids for promoter-*lacZ* fusions		
pPR9TT	Broad-host range vector carries a promoterless *lacZ* gene, Ap^r	Santos et al. (2001)
p*Patu3184-lacZ*	230 bp *atu3184* promoter region containing native Zur box cloned into pPR9TT, Ap^r	This study
p*P#atu3184-lacZ*	230 bp *atu3184* promoter region containing mutated Zur box cloned into pPR9TT, Ap^r	This study
Plasmid for virulence assay		
pCMA1	pTiC58*traM*::*nptII*, Km^r	Hwang et al. (1995)

Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance

genes encoding $YeiR_{Ec}$ and $YjiA_{Ec}$ are not predicted to be regulated by Zur. Many metalloproteins are essential proteins, and their activity is dependent on the presence of a specific metal cofactor; therefore, proper metal allocation and correct metal cofactor loading are crucial for bacterial survival. Members of the COG0523 family are likely to serve this function; however, the metal substrate and the metalloprotein target(s) of most COG0523 subgroups remain to be identified.

A. tumefaciens is a bacterial pathogen that causes the crown gall tumor plant disease. During host-pathogen interactions, they compete for essential metals, including zinc. However, intracellular zinc overload is toxic to cells. To maintain zinc homeostasis, bacteria have evolved mechanisms to acquire and allocate zinc to zinc-containing proteins under zinc limitation and to prevent zinc toxicity mediated by the mismetallation of non-zinc-binding proteins in cases of excessive amounts of zinc through controlling the uptake, export, storage, transfer and reallocation of zinc (Capdevila et al., 2016). In *A. tumefaciens*, Zur has been shown to play a key role in regulating two high-affinity ABC transporters for zinc uptake (TroCBA and ZnuABC) and two zinc chaperones (ZinT and YciC) for the survival of *A. tumefaciens* in a wide range of zinc-limiting conditions (Bhubhanil et al., 2014c; Chaoprasid et al., 2016). The periplasmic ZinT protein likely interacts with TroCBA, which is responsible for the major zinc uptake system, whereas ZnuABC function is independent of ZinT, and ZnuABC seems to play a supporting role when TroCBA is inactive (Chaoprasid et al., 2016). Importantly, even when TroCBA and ZnuABC are still functioning, *A. tumefaciens* requires the functional roles of both periplasmic ZinT and cytoplasmic YciC chaperones because a strain that lacks both *zinT* and *yciC* exhibits a severe growth defect under zinc deprivation (Chaoprasid et al., 2016). The $YciC_{At}$ protein shares high amino acid sequence identity with members of Zur-regulated COG0523, including $CobW1_{Cm}$ (58%), $ZigA_{Ab}$ (55%), $YciC_{Bs}$ (46%) and Zrg_{Cd} (36%), and has low sequence identity with $YjiA_{Ec}$ (19%) and $YeiR_{Ec}$ (18%), which are not regulated by Zur. Similar to $YciC_{Bs}$, the importance of $YciC_{At}$ was revealed when the high-affinity ABC transporter was disrupted (Gaballa and Helmann, 1998; Chaoprasid et al., 2016), although the exact mechanisms and functions of $YciC_{Bs}$ and $YciC_{At}$ are not fully understood. It is possible that these Zur-regulated COG0523 proteins are upregulated under zinc limitation and function as a zinc chaperone that may help transfer and reallocate zinc to selected zinc-requiring target proteins whose functions may be a priority for surviving zinc starvation. Thus far, a mechanism for linking the activity of COG0523 protein to zinc homeostasis has been demonstrated in *A. baumannii* (Nairn et al., 2016). The $zigA_{Ab}$ gene is located adjacent to a cluster of genes encoding the histidine (His) utilization system. Under high His and low bioavailable zinc, $ZigA_{Ab}$ functions as a zinc chaperone required for the His ammonia-lyase HutH, which is involved in His degradation, leading to the release of zinc for cellular demand (Nairn et al., 2016).

The aim of this study was to investigate the molecular mechanism of

regulation and to determine the function of the uncharacterized *atu3184* gene, which is predicted to encode a 347 amino acid protein belonging to the G3E family of P-loop GTPases. The Atu3184 protein contains all of the conserved motifs of the GTPase domain (Haas et al., 2009), including the Walker A (GxxGxGK) and Walker B (ExxG) motifs involved in the binding of the phosphates of nucleotide triphosphate, the TKxD motif for guanine base-binding specificity and the metal-binding GCxCC motif (Fig. S1). Compared to the previously reported members of the G3E family, the Atu3184 protein has the highest amino acid sequence identity with $YjiA_{Ec}$ (27%), followed by $CobW1_{Cm}$ (22%), $ZigA_{Ab}$ (20%), $YciC_{At}$ (19%), Zrg_{Cd} (18%), $YciC_{Bs}$ (17%) and $YeiR_{Ec}$ (17%). Previous comparative computational analysis has predicted the DNA binding sequence of Zur (Zur box) of the *Agrobacterium* group, the 18-bp palindrome 5′-GTAATGTAATAACATTAC-3′ (underlined letters are the center of symmetry of the inverted palindrome) (Panina et al., 2003). The *atu3184* gene is located further downstream of the *yciC* (*atu3181*) gene. During our analysis of the DNA sequence, a potential Zur box (5′-GATATGTTATTACATTAC-3′) was found in the promoter region of *atu3184,* implying that *atu3184* may be an additional target gene of the *A. tumefaciens* Zur regulon. Several mutants involving the inactivation of *atu3184* alone or in combination with zinc transporters and chaperones were generated to assess the possible function of *atu3184*. This information could provide insight to improve the understanding of mechanisms for maintaining zinc homeostasis and how the COG0523 GTPase proteins are linked to the zinc stress response in *A. tumefaciens*.

2. Materials and methods

2.1. Bacterial strains, plasmids, growth conditions and DNA manipulations

The bacterial strains and plasmids used in this study are described in Table 1. The culture conditions, media, and antibiotic concentrations for *A. tumefaciens* and *E. coli* strains were the same as described previously (Nuonming et al., 2018). *A. tumefaciens* and *E. coli* were routinely grown in Luria-Bertani (LB) medium at 28 °C and 37 °C, respectively, with aeration. LA medium is defined as LB medium containing 1.5% agar. DNA manipulations were performed using standard protocols (Sambrook et al., 1989). For DNA cloning, the sequence of inserted DNA was confirmed by sequencing (Macrogen). The primers used in this study are listed in Table S1.

2.2. Construction of pZUR, pATU3184, pPAtu3184 and pP#Atu3184 plasmids

The entire coding region of *zur* (*atu1518*, 414 bp) without its native promoter was amplified with the primers BT3804 and BT982 using genomic DNA of wild-type NTL4 (WT) as a template. The PCR product was then inserted into the SmaI site of pBBR1MCS-2 (Kovach, 1995) to generate the plasmid pZUR for complementation.

The entire coding region of *atu3184* (1,044 bp) without its native promoter was amplified with the primers BT6833 and BT6832 using genomic DNA of WT as a template. The PCR product was then inserted into the SmaI site of pBBR1MCS-4 (Kovach, 1995) to generate the plasmid pATU3184 for complementation.

The 283 bp *atu3184* promoter region was amplified with the primers BT6932 (KpnI) and BT7201 (HindIII) using the genomic DNA of WT as a template. The restriction enzyme sites were incorporated into these primers to allow for fragment ligation into the plasmid vector pBluescript II KS (Stratagene) at the KpnI and HindIII sites, generating the plasmid pPAtu3184 harboring a native Zur box (5′-GATATGTTATTACATTAC-3′).

To mutate the Zur box in the *atu3184* promoter, site-directed mutagenesis was performed using a QuikChange XL mutagenic PCR kit (Stratagene) according to the manufacturer's instructions. The plasmid pPAtu3184 was subjected to site-directed mutagenesis using the primers BT7281 and BT7282 to generate the plasmid pP#Atu3184 harboring a mutated Zur box (5′-GAT**GG**GTTATTACATTAC-3′, the mutation sites are indicated by the bold letters). The mutation was confirmed by DNA sequencing.

2.3. Construction of atu3184 promoter-lacZ fusion plasmids and β-galactosidase activity assay

The promoter region of *atu3184* covering the annotated start codon of *atu3184* was amplified (230 bp, BT6932 and BT6933) and was fused in frame with the *lacZ* reporter gene on the plasmid pPR9TT at the KpnI and HindIII sites generating the plasmid pP*atu3184-lacZ* containing a native Zur box (5′-GATATGTTATTACATTAC-3′).

To construct the *atu3184 promoter-lacZ* fusion that contains a mutated Zur box (5′- GAT**GG**GTTATTACATTAC-3′), the PCR product using the primers BT6932 and BT6933 and the plasmid pP#Atu3184 as a template was fused to *lacZ* on the plasmid pPR9TT at the KpnI and HindIII sites to create the plasmid pP*#atu3184-lacZ*.

A β-galactosidase activity assay was carried out according to a previously reported protocol (Miller, 1972; Bhubhanil et al., 2014a) using log phase cells expressing either pP*atu3184-lacZ* or pP*#atu3184-lacZ* that were grown in LB medium and were treated with 1 mM ethylenediaminetetra acetic acid (EDTA) in the absence or presence of 0.75 mM $ZnCl_2$ for 1 h. The cells were permeabilized by sodium dodecyl sulfate and chloroform. ONPG substrate solution was added, and the OD_{420} was measured. The units of β-galactosidase activity were defined as the change in OD_{420} min^{-1} per OD_{600} of cells. The results were reported as the mean of three biological replicates.

2.4. Construction of A. tumefaciens mutant strains disrupting the atu3184 gene and in combination with other zinc transporters and chaperones

To knock out the *atu3184* gene, the insertional gene inactivation method was used according to a previously reported protocol (Ngoknngam et al., 2009). The PCR fragment (336 bp, BT6798 and BT6799) of the internal region of *atu3184* was amplified and cloned into the suicide plasmid pKNOCK-Gm (Alexeyev, 1999). The resulting recombinant plasmid pKNOCK3184 was then transferred into wild-type NTL4 by electroporation. The *atu3184* mutant strain was selected on an LA plate containing 60 μg/ml gentamicin and was named ZG3184 (*atu3184*::Gm). It should be noted that *atu3184*, *atu3183* and *atu3182* genes are transcribed as a single transcript which was confirmed by reverse transcription PCR analysis, thus, insertional gene inactivation of *atu3184* (*atu3184*::Gm) has a polar effect on the downstream genes *atu3183* and *atu3182* genes (reverse transcription PCR analysis, data not shown).

The plasmid pKNOCKmTROC was constructed using a PCR fragment (293 bp, BT3751 and BT3752) of the internal region of *troC* and cloned into the suicide plasmid pKNOCK-Km (Alexeyev, 1999). The plasmids pKNOCKmYCIC, pKNOCKmZNUA and pKNOCKmZINT were obtained from a previous study (Chaoprasid et al., 2016).

To construct the double mutation strains, including ZG3184TC (*atu3184*::Gm *troC*::Km), ZG3184YC (*atu3184*::Gm *yciC*::Km), ZG3184ZA (*atu3184*::Gm *znuA*::Km) and ZG3184ZT (*atu3184*::Gm *zinT*::Km), the plasmids pKNOCKmTROC, pKNOCKmYCIC, pKNOCKmZNUA and pKNOCKmZINT, respectively, were individually transferred into the ZG3184 strain. The double mutation strains were selected on LA plates containing 60 μg/ml gentamicin and 30 μg/ml kanamycin. All *A. tumefaciens* mutant strains were confirmed by Southern blot analysis.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously described (Bhubhanil et al., 2014b) using RNA extracted from log-phase cells of wild-type NTL4 grown in LB medium and treated with 1 mM EDTA for 15 min. The

junctions between the *atu3182* and *atu3183* genes (BT7108 and BT7107) and between the *atu3183* and *atu3184* genes (BT7106 and BT7127) were amplified with the primer pairs indicated in parentheses.

2.6. Quantitative real-time PCR (qRT-PCR)

Log-phase cells grown in LB medium were either untreated or treated with a metal chelator alone or in combination with a metal for 15 min prior to harvest. EDTA (1 mM) was used as the metal chelator. The metal salts $CdCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_3$, $MnCl_2$, $NiCl_2$ and $ZnCl_2$ were added at a final concentration of 0.75 mM. RNA isolation (Ngokngam et al., 2009) and qRT-PCR analysis (Bhubhanil et al., 2014a) were performed according to previously reported protocols. The primers used for qRT-PCR are listed in Table S1. The amount of a specific target mRNA was normalized to the amount of the housekeeping gene 16S rRNA. The relative expression was reported using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7. Protein expression and purification of the recombinant Zur-Strep-tagged protein (rZur)

To produce *Strep*-tag II (a short peptide consisting of eight amino acids, WSHPQFEK) that is fused in frame to the C-terminus of Zur (rZur), the primers BT6173 and BT6174, which contain a BsaI site, were used to amplify the coding region of *zur*. The PCR products digested with BsaI were then cloned into BsaI-digested pASK-IBA3 (IBA, Germany), creating the plasmid pZur-Strep-tag. The rZur protein was overproduced in *E. coli* DH5< and purified using a *Strep*-Tactin Sepharose column according to previously reported protocols (Dokpikul et al., 2016), and EDTA was omitted in all buffers during protein purification. The purity of the rZur protein was analyzed by SDS-PAGE and Coomassie blue staining (Fig. S2).

2.8. Electrophoretic mobility shift assay (EMSA)

A ^{32}P-labeled DNA probe containing a 283 bp PCR fragment (BT6898 and BT6899) of the *atu3184* promoter was prepared, and EMSA was carried out according to procedures that were described previously (Dokpikul et al., 2016). In the binding reactions, the purified rZur and DNA probe were incubated in 1 × buffer [20 mM Tris-HCl pH 7, 50 mM KCl, 1 mM dithiothreitol (DTT), 5% glycerol, 0.5 μg/ml calf thymus DNA, and 0.05 mg/ml bovine serum albumin (BSA)] containing 1.5 mM EDTA either with or without metal addition ($CdCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_3$, $MnCl_2$, $NiCl_2$ or $ZnCl_2$ at the indicated concentrations in the figure legend).

The native *atu3184* promoter probe (Zur box, 5′-GATATGTTATTA CATTAC-3′) and the mutated *atu3184* promoter probe (mutated Zur box, 5′-GAT**GG**GTTATTACATTAC-3′) were amplified with the primers BT6898 and BT6899 (283 bp) using the plasmids pPAtu3184 and pP#Atu3184, respectively, as a DNA template.

The 373 bp PCR fragment (BT4946 and BT4947) containing the multiple cloning site (MCS) region of the plasmid vector pBBR1MCS-4 (Kovach, 1995) was labeled with ^{32}P and used as a negative control probe.

2.9. Identification of the atu3184 transcriptional start site

Log-phase WT cells grown in LB and treated with 1 mM EDTA for 15 min were harvested, and RNA isolation and 5′ rapid amplification of cDNA ends (5′ RACE) were performed to determine the transcriptional start site of *atu3184* using a 5′ RACE kit (Roche) according to the manufacturer's instructions with the primers SP1 (BT6902) and SP2 (BT6903).

2.10. DNase I footprinting assay

The binding reaction, DNase I digestion and separation on an 8% polyacrylamide and 8 M urea sequencing gel were carried out as described previously (Dokpikul et al., 2016). The purified rZur protein (0.1, 0.5, 1 and 5 μM) was incubated with the DNA fragments (245 bp, BT6898 and BT6927) containing the *atu3184* promoter in which the ^{32}P-end labeled either the top or bottom strand. Dideoxy DNA sequencing of the DNA fragment was also performed using either the primer BT6898 or BT6927, and the samples were run alongside the DNase I footprint.

2.11. Sensitivity to EDTA

The EDTA sensitivity test was performed according to a previously described method (Chaoprasid et al., 2016). Log-phase cells grown in LB were adjusted by 10-fold serial dilutions. Five microliters from each dilution were spotted on AB medium (Cangelosi et al., 1991) agar plates either with or without EDTA (0.3, 0.6, 0.8 and 1.0 mM) and incubated at 28 °C for 2 days. The experiments were repeated at least two times to ensure reproducibility.

2.12. Quantification of total cellular zinc content by inductively coupled plasma mass spectrometry (ICP-MS)

Samples were prepared from cells grown in LB supplemented with 500 μM EDTA at 28 °C for 24 h, and zinc ions were measured in parts per billion (ppb) as previously described (Bhubhanil et al., 2014a). The data were reported as the means of biological triplicates.

2.13. Virulence assay

The ability of the *A. tumefaciens* strains to infect *Nicotiana benthamiana* plants and form tumors was monitored using a previously described protocol (Murashige and Skoog, 1962; Kitphati et al., 2007). Cells carrying the pCMA1 plasmid were adjusted to an OD_{600} of 0.05 in hormone-free MS liquid medium (Murashige and Skoog, 1962). The cell suspensions were cocultivated with 0.5-cm squares of *N. benthamiana* leaf pieces at room temperature for 10 min. Thirty leaf squares were tested for each bacterial strain. Leaf pieces incubated in hormone-free MS medium without bacterial cells were used as a negative control. The tumors on each leaf piece were observed after 4 weeks.

3. Results

3.1. Analysis of the Zur-regulated operon consisting of atu3184, atu3183 and atu3182 genes

The *A. tumefaciens troCBA* operon (*atu3180*, *atu3179* and *atu3178*, respectively) and *yciC* (*atu3181*) have been previously reported as Zur targets (Fig. 1A) (Chaoprasid et al., 2016). The *troCBA* operon encodes a zinc uptake system consisting of an ATP-binding protein (TroC), a permease (TroB) and a periplasmic substrate-binding protein (TroA). YciC is a cytoplasmic zinc chaperone. Inspection of the DNA sequences of the genes downstream of *yciC*, including *atu3182*, *atu3183* and *atu3184* (Fig. 1A), revealed that these genes are likely to form an operon. The *atu3182* and *atu3183* genes are separated by 97 bp. The stop codon **TG**A of *atu3184* overlaps (bold letters) with the start codon A**TG** of *atu3183*. The *atu3184* gene encodes a protein (347 amino acids) in the G3E family of P-loop GTPase, while *atu3183* (89 amino acids) and *atu3182* (145 amino acids) encode proteins with unknown functions (the Kyoto Encyclopedia of Genes and Genomes annotation, http://www.genome.jp/kegg). Furthermore, we identified a potential Zur box, 5′-GATATGTTATTACATTAC-3′, which is located preceding *atu3184*, implying that this gene may be regulated by Zur.

First, reverse transcription polymerase chain reaction (RT-PCR) was

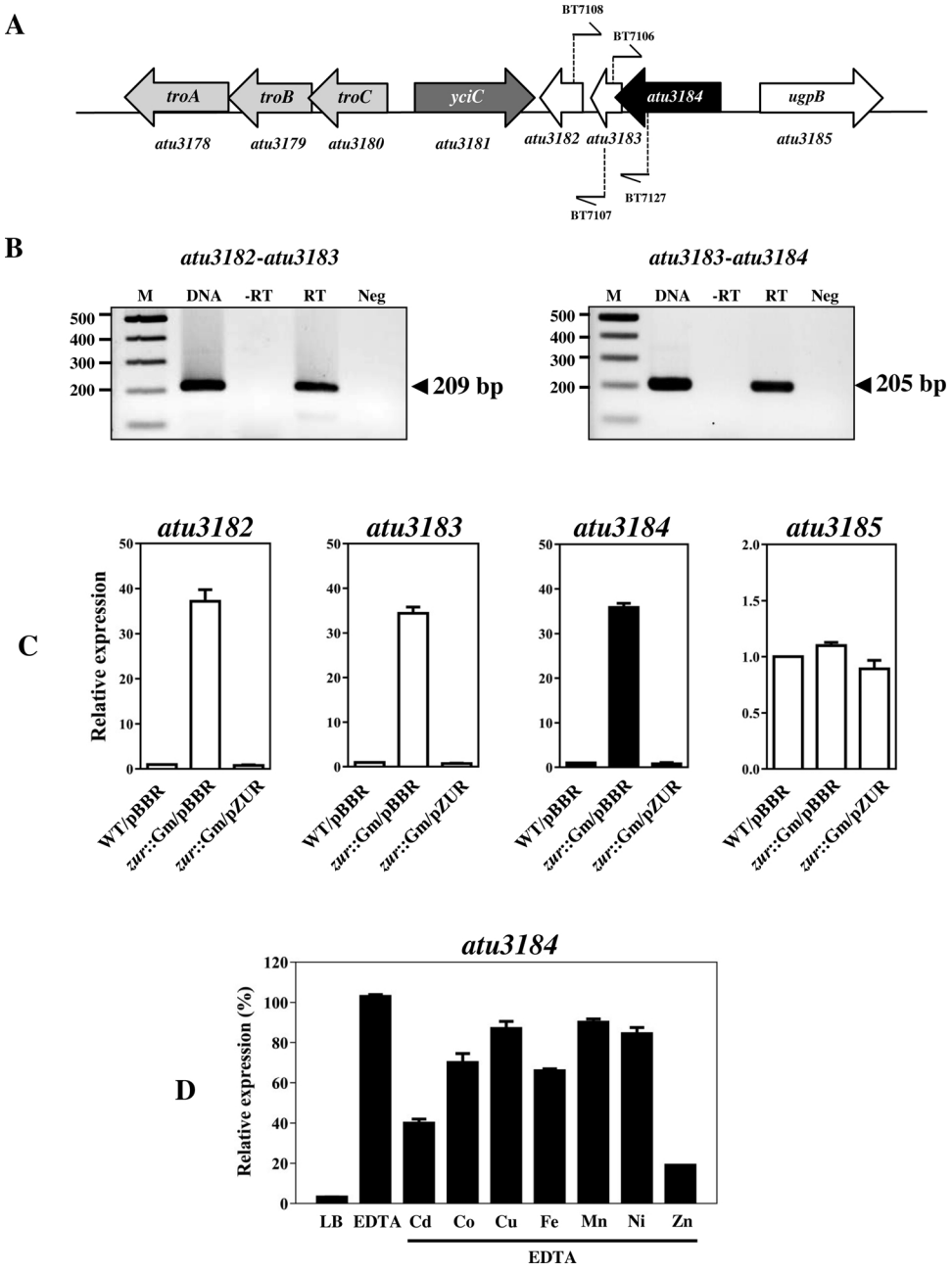


Fig. 1. (A) A genetic map of the *atu3184-atu3183-atu3182* operon and adjacent genes in *A. tumefaciens*. The locations of the primers (BT7108, BT7107, BT7106 and BT7127) used in the reverse transcription PCR (RT-PCR) analysis are indicated by arrows.
(B) Reverse transcription polymerase chain reaction (RT-PCR) analysis showing that the *atu3184*, *atu3183* and *atu3182* genes are co-transcribed. The intergenic regions of *atu3182-atu3183* (BT7108 and BT7106) and *atu3183-atu3184* (BT7106 and BT7127) were amplified using the primer pairs indicated in parentheses. The expected sizes of the PCR products are indicated by triangles. Lane M: 100 bp DNA ladder. Lane RT: a DNase I-treated RNA sample prepared from the wild-type NTL4 strain was then treated with reverse transcriptase (RT) to produce cDNA. A DNase I-treated RNA sample without reverse transcription (-RT) served as a control for DNA contamination. Reactions with and without a genomic DNA template were performed and used as a positive control (DNA) and a negative control (Neg), respectively.
(C) Quantitative real-time PCR (qRT-PCR). The relative expression values represent the fold changes in the expression of *atu3182*, *atu3183*, *atu3184* and *atu3185* in the *zur* mutant strain (*zur*::Gm) grown in LB medium compared to the wild-type NTL4 strain (WT). pBBR is a multicopy plasmid vector, while pZUR carries a functional *zur* gene. The expression levels of *atu3182*, *atu3183*, *atu3184* and *atu3185* are relative to that of the WT/pBBR strain (regarded as 1). The results represent the means of triplicate independent samples ± the standard deviation.
(D) qRT-PCR analysis of metal suppression of EDTA-induced expression of *atu3184* in WT cells. The cells were grown in LB medium and under metal-limited conditions (LB + 1 mM EDTA). A metal salt ($CdCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_3$, $MnCl_2$, $NiCl_2$ or $ZnCl_2$) was added to a final concentration of 0.75 mM. The expression levels are shown as a percentage and are relative to those in cells grown in LB + 1 mM EDTA (100%). The results represent the means of triplicate independent samples ± the standard deviation.

performed (Fig. 1B), and the results confirmed that *atu3184*, *atu3183* and *atu3182* genes were transcribed as a single transcript in which *atu3184* is the first gene of the operon. Next, to investigate whether *atu3184* is regulated by Zur, quantitative real-time PCR (qRT-PCR) was performed to measure the levels of *atu3184* transcription in the *zur* mutant strain (SPP12, *zur*::Gm) (Bhubhanil et al., 2014c). Compared to the levels in the wild-type strain (WT, WT/pBBR), increased expression of *atu3184* (˜35-fold) was observed in the *zur* mutant strain (*zur*::Gm/pBBR) and could be suppressed in the complemented strain when a functional *zur* gene was provided in trans by the multicopy plasmid pZUR (*zur*::Gm/pZUR, Fig. 1C). Similar patterns were observed for the expression of *atu3183* and *atu3182*, which is consistent with the interpretation that *atu3184*, *atu3183* and *atu3182* are cotranscribed. In contrast to the expression of the *atu3185* transcript, a gene (*ugpB*) encoded a glycerol-3-phosphate ABC transporter substrate-binding protein and was located next to *atu3184* with a divergent transcriptional direction and was not affected when *zur* was disrupted (Fig. 1C). These results suggest that Zur is a repressor of the *atu3184-atu3183-atu3182* operon.

A. tumefaciens Zur-regulated genes have been shown to be inducible under metal-limited growth conditions imposed by the addition of the metal chelator EDTA and have been found to be more specific for zinc deprivation (Chaoprasid et al., 2016). qRT-PCR analysis revealed the induction of *atu3184* by approximately 35-fold (expression level regarded as 100%) when WT cells were treated with 1 mM EDTA (Fig. 1D). The addition of metals (0.75 mM of $CdCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_3$, $MnCl_2$, $NiCl_2$ or $ZnCl_2$) suppressed the EDTA induction of *atu3184*, in which zinc was shown to be the best suppressor (Fig. 1D). These results demonstrated that the response of *atu3184* was likely more specific to zinc limitation.

3.2. The rZur protein binds to the atu3184 promoter in the presence of zinc in vitro

The derepression of *atu3182*, *atu3183* and *atu3184* was observed in the *zur* mutant (Fig. 1C), suggesting that Zur could be involved in the

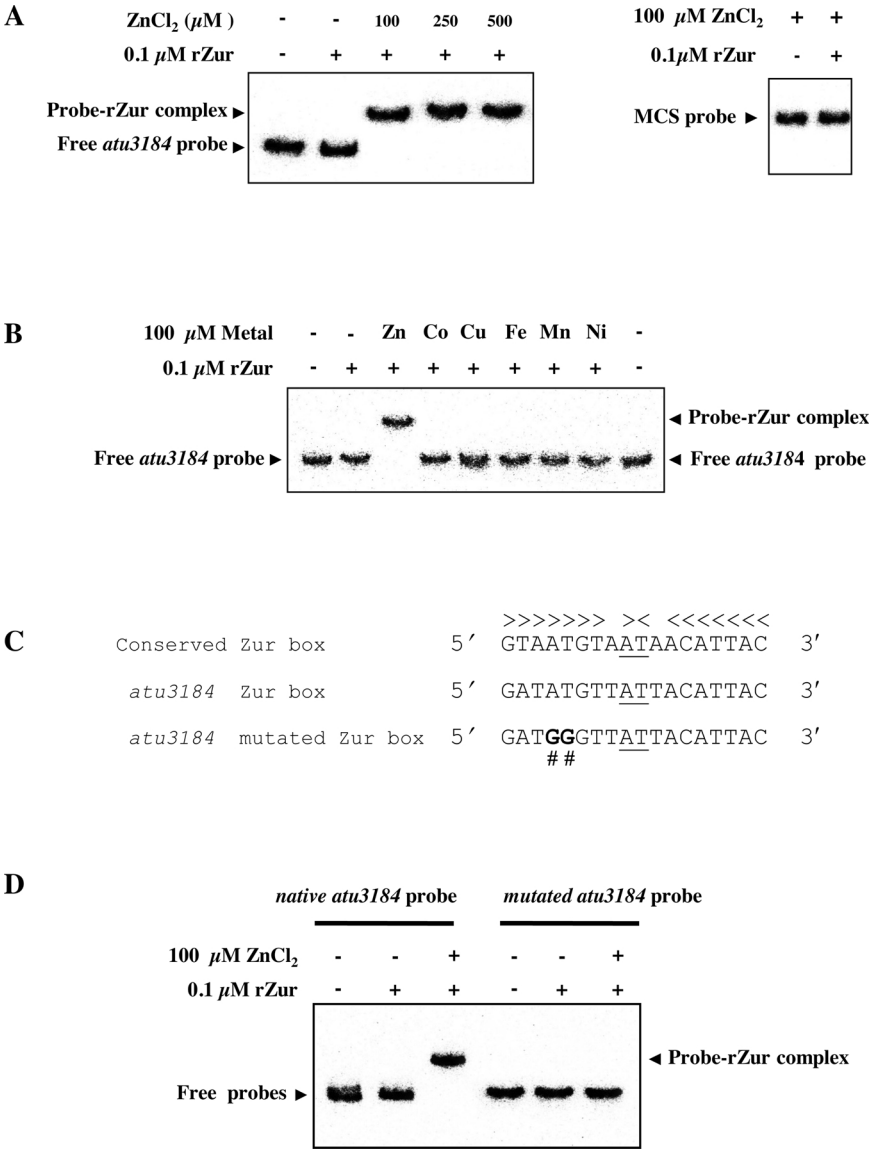


Fig. 2. (A) *In vitro* binding of rZur to the *atu3184* promoter. An electrophoretic mobility shift assay (EMSA) was carried out in binding buffer containing 1.5 mM EDTA. The rZur protein and *atu3184* promoter probe (283 bp, BT6898 and BT6899) were incubated either with or without increasing concentrations of $ZnCl_2$ (100, 250 and 500 μM). The bands of the free *atu3184* probe and probe-rZur complex are indicated by triangles. The MCS probe (373 bp DNA fragment without a potential Zur box) was used as a negative control probe.
(B) The effect of metals on the ability of rZur to bind the *atu3184* promoter probe. EMSA was carried out as mentioned above, and a metal salt ($CdCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_3$, $MnCl_2$, $NiCl_2$ or $ZnCl_2$) was added in the binding reaction to a final concentration of 100 μM.
(C) The conserved Zur box sequence for the *Agrobacterium* group (Panina et al., 2003). The underlined nucleotides A and T indicate the center of symmetry of the inverted palindrome. The inverted sequences are indicated by angled brackets. The potential Zur box was identified in the *atu3184* promoter region. The mutated nucleotides in the *atu3184* Zur box are indicated by bold letters with #.
(D) EMSA showing that the rZur protein did not bind to the mutated Zur box. The ability of the rZur protein to bind to the mutated *atu3184* probe (283 bp) containing a mutation in the Zur box (5′- GAT**GG**GTTATTACATTAC-3′) was compared to its binding of the native *atu3184* probe (283 bp, BT6898 and BT6899, a positive control probe containing the Zur box 5′-GATATGTTATTACATTAC-3′). Free probes and the probe-rZur complex are indicated by triangles.

direct or indirect control of *atu3184-atu3183-atu3182* transcription. Whether Zur meditated the repression of the *atu3184-atu3183-atu3182* operon through Zur binding directly to the *atu3184* promoter was tested *in vitro*. The recombinant Zur (rZur, C-terminal Strep-tagged fusion) protein was purified (Fig. S2 A), and its binding ability to the *atu3184* promoter probes labeled with ^{32}P was monitored using an electrophoretic mobility shift assay (EMSA) (Fig. 2). In the absence of metal, the rZur protein (0.1 μM) did not retard the mobility of the *atu3184* promoter probe (Fig. 2A). Zur proteins from many bacteria require zinc as a cofactor for their ability to bind DNA (Mikhaylina et al., 2018). When $ZnCl_2$ was added into the binding reactions at concentrations of 100, 250 or 500 μM, a single shifted band (probe-rZur complex) was observed, demonstrating the binding of the rZur protein to the *atu3184* promoter probe (Fig. 2A). However, other metal salts, such as $CoCl_2$, $CuSO_4$, $FeCl_3$, $MnCl_2$ and $NiCl_2$, did not facilitate the formation of the probe-rZur complex (Fig. 2B). At 100 μM $ZnCl_2$, the interaction of rZur with the *atu3184* promoter probe using various concentrations of rZur (0.005, 0.01, 0.05, 0.1, 0.5, 1 and 2 μM) was also determined (Fig. S2B), and the shifted band was observed starting at 0.005 μM rZur. In contrast, the shifted band was not observed when 0.1 μM rZur protein was incubated with the MCS probe [a negative control probe containing DNA sequences of multiple cloning sites (MCS) from the plasmid vector pBBR1MCS-4 (Kovach et al., 1995), without a potential Zur Box] in the reaction containing 100 μM $ZnCl_2$ (Fig. 2A). Even when the concentrations of either the rZur protein (0.5 and 1 μM) or $ZnCl_2$ (250 and 500 μM) increased in the binding reactions of the MCS probe, the retarded band was not detected (data not shown). These results indicated that the interaction between the rZur protein and the *atu3184* promoter probe was likely to be sequence specific and metal-cofactor specific.

To test whether the potential Zur box, 5′-GATATGTTATTACAT TAC-3′, is involved in the interaction with Zur, an *atu3148* probe containing a mutated Zur box, 5′- GAT**GG**GTTATTACATTAC-3′ (the mutation sites are indicated by the bold letters), was generated (Fig. 2C). In contrast to the native *atu3184* control probe, the result of EMSA, shown in Fig. 2D, shows that the retarded band (probe-rZur complex) was not detected when rZur was incubated with the mutated *atu3184* probe. Even when the binding reactions containing increased concentrations of rZur (0.5, 1 and 2 μM), the retarded band was not detected (data not shown). This evidence suggested that the 5′-GATATG TTATTACATTAC-3′ sequence in the *atu3184* promoter is required for rZur binding.

To map the promoter of *atu3184*, 5′ rapid amplification of cDNA ends (5′-RACE) was performed to determine the transcriptional start site of *atu3184,* which was found to be at the G residue located 98 bp upstream of the predicted start codon GTG of *atu3184* (Fig. 3A). The

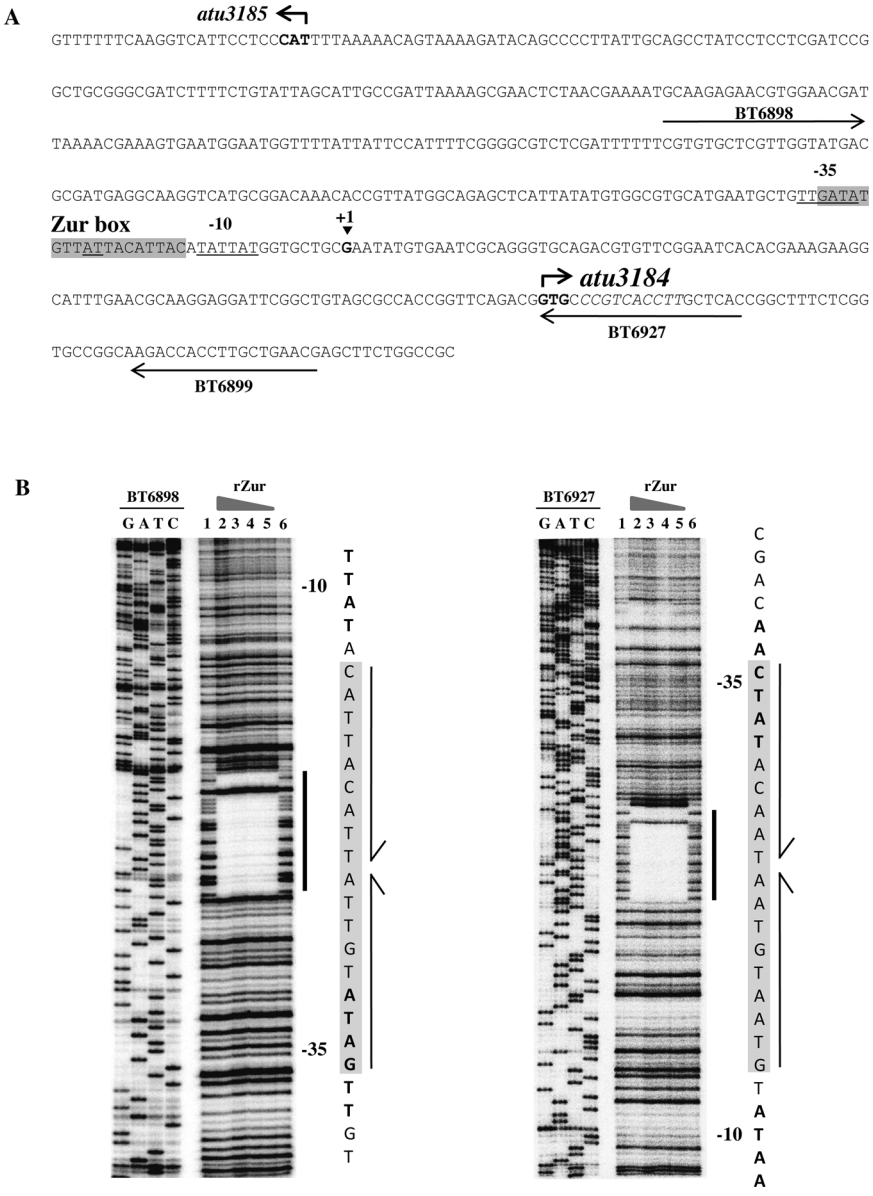


Fig. 3. (A) The nucleotide sequence of the *atu3184* promoter region. The annotated start codons for *atu3184* and *atu3185* are indicated by bold letters, and the bent arrows indicate the direction of transcription. The transcriptional start site (+1) of *atu3184* at the G residue is depicted by a triangle. The proposed -10 (TATTAT) and -35 (TTGATA) sequences are underlined. The Zur box (5′-GATATGTTATTACATTAC-3′) is highlighted on the gray background. The positions of the primers (BT6898, BT6899 and BT6927) are indicated by arrows.
(B) Identification of the Zur recognition sequence by DNase I footprinting analysis. The 245 bp DNA fragments containing the *atu3184* promoter were ^{32}P-end-labeled on either the top (BT6898) or the bottom (BT6927) strand and incubated without (lanes 1 and 6) or with various concentrations of the purified rZur protein (lanes 2 to 5: 5, 1, 0.5 and 0.1 μM, respectively). The protected areas from DNase I digestion are indicated by solid lines, and the nucleotide sequences are shown alongside. The half-headed arrows indicate the inverted repeat of the Zur box. The proposed -10 and -35 sequences are indicated by bold letters. Lanes G, A, T and C are the dideoxy DNA sequencing of the 245 bp DNA fragment using either the primer BT6898 or BT6927.

potential promoter sequences recognized by σ^{70}-RNA polymerase (Harley and Reynolds, 1987), -10 (TATTAT) and -35 (TTGAAT) sequences separated by 15 bp were proposed for the *atu3184* promoter (Fig. 3A). The Zur box (5′-GATATGTTATTACATTAC-3′) was found to be located overlapping with the putative -35 sequence (Fig. 3A), in accordance with the view that Zur acts as a repressor that inhibits the transcription of target genes by interfering with RNA polymerase binding (Mikhaylina et al., 2018). The specific DNA recognition sequence for rZur binding was further confirmed by a DNase I footprinting assay (Fig. 3B). The protected regions, approximately 27 and 29 nucleotides spanning the Zur box (5′-GATATGTTATTACATTAC-3′), were detected for the top and bottom strands, respectively (Fig. 3B).

3.3. In vivo measurements of the activity of the atu3184 promoter and regulation by Zur

To confirm the promoter activity, a 230 bp promoter region of *atu3184* was fused to a *lacZ* reporter gene (Fig. 4A). In WT cells carrying p*Patu3184-lacZ*, β-galactosidase activity was inducible when the cells were treated with 1 mM EDTA, and EDTA induction was suppressed when 0.75 mM $ZnCl_2$ was added, demonstrating that the activity of the *atu3184* promoter is responsive to zinc levels. Consistent with the qRT-PCR results (Fig. 1C), the promoter activity of *atu3184* (p*Patu3184-lacZ*), which was determined by measuring β-galactosidase activity, was derepressed in the *zur* mutant strain (*zur*::Gm) compared to the wild-type (WT) strain (Fig. 4B). This *in vivo* reporter assay further confirmed that *atu3184* expression is negatively regulated by Zur. When the Zur box in the promoter region of *atu3184* was mutated (5′-GAT**GG**GTTATTACATTAC-3′, p*P#atu3184-lacZ*), the WT strain showed high levels of β-galactosidase activity in all tested conditions, demonstrating the loss of zinc-responsive regulation by Zur (Fig. 4B). These results suggested that the Zur box (5′-GATATGTTATTACATTAC-3′) is required for Zur-mediated repression of the *atu3184* promoter.

3.4. Investigation of the role of atu3184 in response to metal limitation

Induction of *atu3184* expression occurred under metal-limiting conditions imposed by the divalent metal chelator EDTA (Fig. 1D). To directly determine the function of *atu3184* in response to metal deprivation, a mutant strain (ZG3184, *atu3184*::Gm) disrupting the *atu3184* gene was constructed. The growth of WT and *atu3184*::Gm cells in the presence of various concentrations of EDTA was compared. The growth

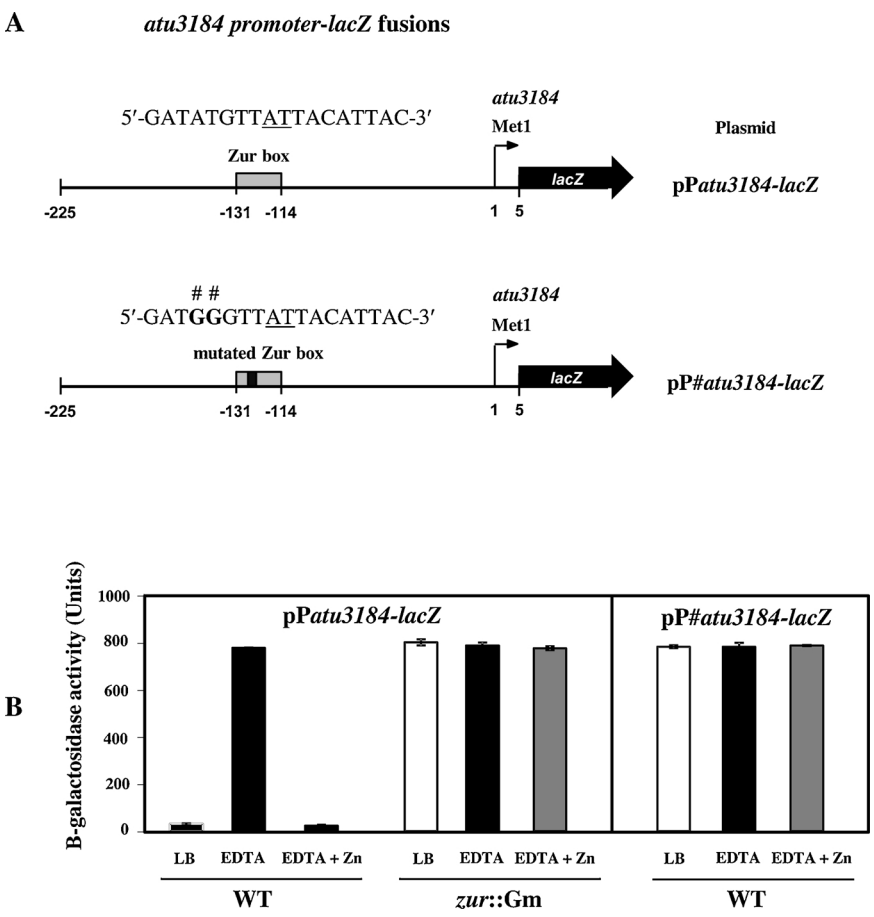


Fig. 4. (A) Schematic representation of the *atu3184* promoter-*lacZ* fusions (not drawn to scale). The plasmid pP*atu3184-lacZ* contains the *atu3184* promoter (230 bp, BT6932 and BT6933) with a native Zur box (5′-GATATG TTATTACATTAC-3′). The numbers indicate the positions relative to the G residue of the annotated *atu3184* GTG start codon (Met1). The bent arrow indicates the transcriptional direction. The plasmid pP*#atu3184-lacZ* contains the *atu3184* promoter with a mutated Zur box (5′-GAT**GG**GTTATTACATTAC-3′). The mutation sites are indicated by bold letters and marked with #.
(B) Zur regulates *atu3184* promoter activity. Log phase cells were grown in LB and either untreated or treated with 1 mM EDTA in the absence or presence of 0.75 mM $ZnCl_2$. β-galactosidase activity was measured from wild-type (WT) and *zur* mutant (*zur*::Gm) strains carrying either the plasmid pP*atu3184-lacZ* or pP*#atu3184-lacZ*. The results represent the means and standard deviations of three independent experiments.

of *atu3184*::Gm cells was similar to that of WT cells on AB medium plates containing 0.6, 0.8 and 1 mM EDTA (Fig. 5A & B). Consistent with a previous study (Chaoprasid et al., 2016), the inactivation of either the periplasmic zinc chaperone ZinT (*zinT*::Gm, Fig. 5A) or the zinc uptake TroC (*troC*::Gm, Fig. 5B) caused cells to become hypersensitive to EDTA, while the single inactivation of the cytoplasmic zinc chaperone YciC (*yciC*::Km, Fig. 5A) and the zinc uptake ZnuA (*znuA*::Gm, Fig. 5B) had no apparent effect.

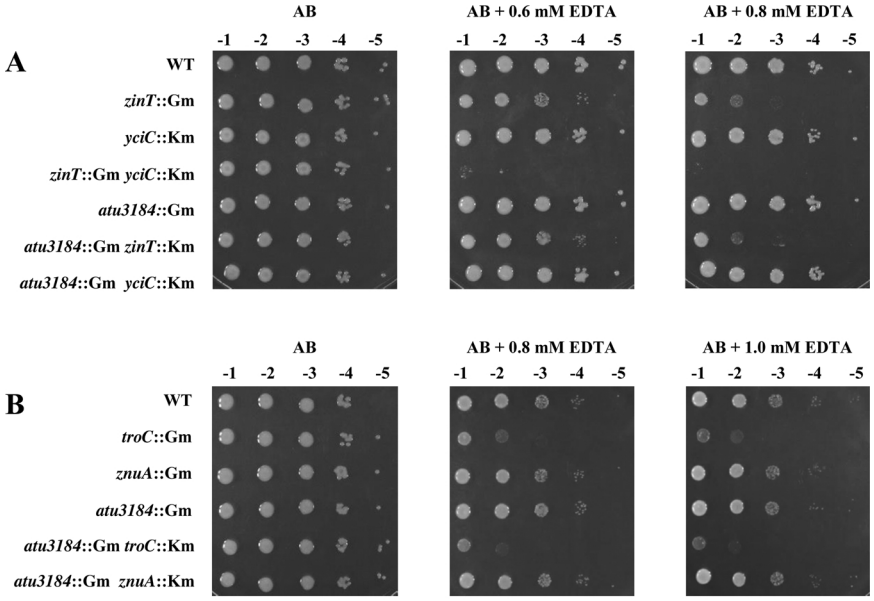
Similar to YciC, the Atu3184 protein belongs to the COG0523 family. Although the single inactivation of *yciC* did not affect cell growth, a previous report (Chaoprasid et al., 2016) showed that inactivation of *yciC* in combination with *zinT* (*zinT*::Gm *yciC*::Km) led to a severe growth defect under metal-limited conditions that were seen at low concentrations of EDTA (AB + 0.6 mM EDTA, Fig. 5A). Therefore, the role of YciC as a zinc chaperone was proposed (Chaoprasid et al., 2016). Next, strains carrying mutations at *atu3184* in combination with other zinc chaperones (*zinT* and *yciC*) or zinc importers (*troC* and *znuA*) were generated to further evaluate the functional role of *atu3184* with these zinc-responsive genes. Unlike YciC, the Atu3184 protein may play a minor role in zinc acquisition, or its function may differ from YciC. It was found that the mutation of *atu3184* did not increase the EDTA sensitivity of the double mutant strains, including the *atu3184*::Gm *zinT*::Km, *atu3184*::Gm *yciC*::Km, *atu3184*::Gm *troC*::Km, and *atu3184*::Gm *znuA*::Km strains, compared with the single mutant strains *zinT*::Gm, *yciC*::Km, *troC*::Gm and *znuA*::Gm, respectively (Fig. 5A & B).

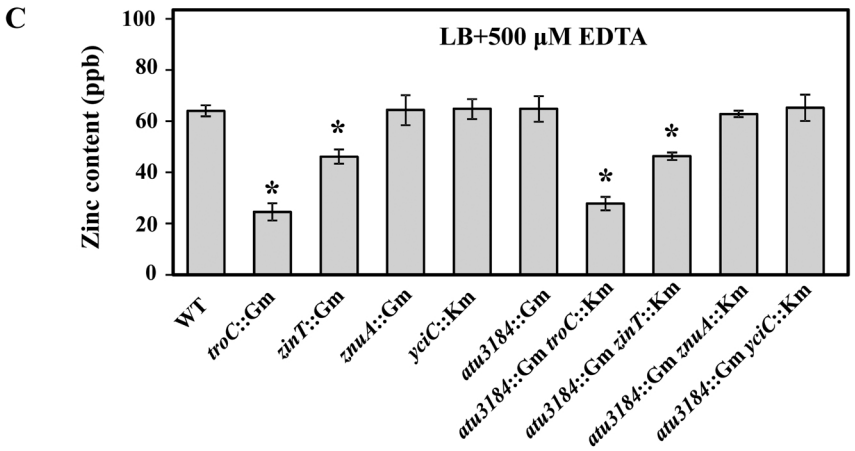
Furthermore, *A. tumefaciens* YciC function could not be substituted by the Atu3184 protein. Consistent with the previous study, expressing the functional *yciC* from the plasmid pYCIC could restore the growth defect of the double mutant strains *zinT*::Gm *yciC*::Km and *troC*::Gm *yciC*::Km (Chaoprasid et al., 2016) back to levels similar to single mutant strains *zinT*::Gm and *troC*::Gm at 0.3 and 0.6 mM EDTA, respectively (Fig. S3). In contrast, expressing the functional *atu3184* from the plasmid pATU3184 could not reverse the EDTA-hypersensitive phenotype of the double mutant strains (Fig. S3).

A previous study has shown a correlation between decreased total cellular zinc content and the EDTA-hypersensitive phenotype of *A. tumefaciens* strains that lack genes encoding zinc importers and chaperones (Chaoprasid et al., 2016). ICP-MS analysis was also performed using cells grown under metal limitation (LB plus 500 μM EDTA, which did not affect the growth of all tested strains) to test whether cellular zinc content was affected by the disruption of *atu3184*. The single mutant *znuA*::PGm, *yciC*::Km and *atu3184*::Gm strains and the double mutant *atu3184*::Gm *znuA*::Km and *atu3184*::Gm *yciC*::Km strains showed zinc levels similar to that of WT (Fig. 5C), which was consistent with the observation that these mutant and WT strains showed similar growth with EDTA sensitivity (Fig. 5A & B). In contrast to the control strains reported in a previous study (Chaoprasid et al., 2016), the *troC*::Gm and *zinT*::Gm strains contained less zinc than the WT strain (*troC*::Gm < *zinT*::Gm < WT, Fig. 5C) and were hypersensitive to EDTA (Fig. 5A & B). The additional mutation of *atu3184* in these control background strains (*atu3184*::Gm *troC*::Km and *atu3184*::Gm *zinT*::Km) did not further reduce cellular zinc content (Fig. 5C), which was also correlated with the phenotype of EDTA sensitivity (Fig. 5A & B). Overall, the results of the ICP-MS analysis suggested that the inactivation of *atu3184* had no effect on the total cellular zinc content. Furthermore, the levels of other metals, such as copper, iron, manganese and nickel, in *atu3184*::Gm and WT strains were not significantly different (data not shown).

3.5. Ability of the atu3184 mutant to form tumors on plant

The virulence of the *A. tumefaciens* strain lacking the *atu3184* gene (*atu3184*::Gm) was assessed by infecting a host plant, *Nicotiana benthamiana*. Tumor formation was observed in all leaf pieces infected by





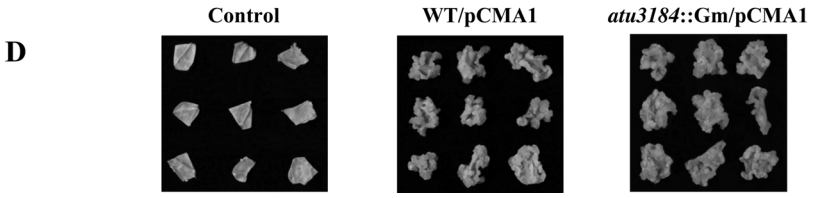


Fig. 5. (A) and (B) Sensitivity to EDTA. The *A. tumefaciens* strains included wild-type NTL4 (WT) and the following mutant strains: PC135 (*zinT*::Gm); YC154 (*yciC*::Km); ZTYC15 (*zinT*::Gm *yciC*::Km); ZTYC15 (*zinT*::Gm *yciC*::Km); ZG3184 (*atu3184*::Gm); ZG3184ZT (*atu3184*::Gm *zinT*::Km); ZG3184YC (*atu3184*::Gm *yciC*::Km); TC142 (*troC*::Gm); PS132 (*znuA*::Gm); ZG3184TC (*atu3184*::Gm *troC*::Km); and ZG3184ZA (*atu3184*::Gm *znuA*::Km). The cells were adjusted, serially diluted, and spotted onto plates containing AB or AB with EDTA (0.6, 0.8 and 1.2 mM). Ten-fold serial dilutions are indicated. The plates were incubated at 28 °C for 2 days.
(C) Total cellular zinc content in WT and mutant strains determined using ICP-MS. The cells were grown in LB with 0.5 mM EDTA for 24 h. The data are reported as the means of biological triplicates. The error bars indicate the standard deviations. The bars marked with asterisks are significantly different from the WT strain ($P < 0.05$ using an unpaired Student's *t*-test).
(D) Virulence assay. Leaf squares of *Nicotiana benthamiana* plant were infected with WT and mutant *atu3184*::Gm cells carrying the pCMA1 plasmid. Leaf pieces without bacterial infection were used as a negative control. Tumor formation was observed after 4 weeks. Representative leaf pieces (from $n = 30$) are shown.

the mutant *atu3184*::Gm strain, similar to those infected with the wild-type strain (Fig. 5D). It was observed that the *atu3184* gene might not be an essential virulence factor for infection, particularly under the tested condition and plant host. A more quantitative plant assay may be required to reveal a subtle effect of *Atu3184* mutation on *A. tumefaciens* virulence.

4. Discussion

The *A. tumefaciens* Zur (Zur_{At}) protein regulates zinc homeostasis by repressing two high-affinity ABC transporters for zinc uptake (TroCBA and ZnuABC) and two zinc chaperones (ZinT and YciC) in response to high zinc concentrations (Chaoprasid et al., 2016). All of these Zur-regulated genes harbor a potential Zur binding consensus sequence (Zur box) in their promoter regions (Bhubhanil et al., 2014c; Chaoprasid et al., 2016). However, the direct binding of Zur to the predicted Zur boxes has not been experimentally demonstrated in *A. tumefaciens*. In this study, an additional member of the *A. tumefaciens* Zur regulon, the *atu3184, atu3183 and atu3182* operon, was identified. Furthermore, the interaction of Zur_{At} with a Zur box was shown, which is useful for better understanding of the molecular basis of Zur regulation in alphaproteobacteria, such as *A. tumefaciens*.

Currently, three crystal structures of the Zur protein from the *Mycobacterium tuberculosis* Zur (Zur_{Mt}) (Lucarelli et al., 2007), the *Streptomyces coelicolor* Zur (Zur_{Sc}) (Shin et al., 2011) and the *E. coli* Zur (Zur_{Ec}) (Gilston et al., 2014), have been reported. The Zur_{At} protein shares some amino acid sequence identity with Zur_{Ec} (29%), Zur_{Mt} (21%) and Zur_{Sc} (18%) (Fig. S4 A). The monomer of Zur proteins has

two domains: an N-terminal DNA-binding domain and a C-terminal dimerization domain (Fig. S4 A). The two domains are connected by a hinge. The monomer of Zur_{Ec} contains two zinc-binding sites (Gilston et al., 2014), while the monomers of Zur_{Mt} and Zur_{Sc} (sites C, M and D) contain three zinc-binding sites (Lucarelli et al., 2007; Shin et al., 2011). Structural Zn site 1 (site C) is highly conserved and consists of two CxxC motifs located in the dimerization domain (Fig. S4 A). Site 2 (site M) is the Zn sensory site in which the Zn ion is coordinated by residues from both domains and the hinge region. These coordinating residues for site 2 in Zur_{Ec} (H77, C88, H96 and E111) are different from those in Zur_{Mt} (D62, C76, H81 and H83) and Zur_{Sc} (D65, C79, H85 and H87) (Fig. S4 A). Site 3 (site D) is an additional Zn sensory site found within the dimerization domain of Zur_{Mt} (H79, H81, E101 and H118) and Zur_{Sc} (H84, H86, E105 and H122) (Fig. S4 A). The regulatory role of site 3 has been confirmed for Zur_{Sc} and has been demonstrated to be necessary for the repression of some sensitive target genes (Shin et al., 2011). The activity of Zur_{Sc} is modulated by two regulatory Zn sites (sites M and D), providing an advantage for the graded expression of zinc-responsive genes to survive a broader range of zinc-limited environments (Shin et al., 2011). The analysis of the amino acid sequence of Zur_{At} revealed that Zur_{At} has a conserved structural Zn site 1 (C92, C95, C132 and C135) and a Zn sensory site 2 (H61, C72, C80 and E100), similar to those of Zur_{Ec} (Fig. S4 A).

Among the three crystal structures of Zur that are currently available, Zur_{Ec} is the only Zur protein that has been crystallized in complex with its cognate DNA (a 31 bp duplex derived from the Zur box of the *znuABC* promoter, 5′-AGAAGTGTGATATTATAACATTTCATGACT ATG-3′) (Fig. S4B). This study provided insights into which residues are critical for the Zur-DNA interaction (Gilston et al., 2014). The amino acid residues for DNA interactions were shown to be quite conserved among Zur proteins (indicated with •, Fig. S4 A). It was found that Y45 and R65 are the key residues of Zur_{Ec} for the interaction with Zur box purines (Gilston et al., 2014). The R65 residue is conserved and critical for binding to DNA by members of the Fur family to which Zur belongs, whereas the Y45 residue is unique to Zur and contacts the inner bases (TATA) of the Zur box (Gilston et al., 2014). It has been proposed that Y45 is a signature residue that facilitates the differential recognition sequences of Zur and Fur in *E. coli* (Gilston et al., 2014). Zinc occupancy at structural site 1 stabilizes the Zur dimer interface. The Zur dimer can adopt either closed or open conformations (Mikhaylina et al., 2018). In contrast to the open conformation, the closed conformation of dimeric Zur has a high DNA binding affinity. When Zn is loaded at Zn sensory site 2, this results in a further increase in the stabilization of the closed DNA binding conformation of the Zur_{Ec} dimer (Gilston et al., 2014). Zur_{Ec} binds to the *znuABC* promoter as a dimer of dimers (Gilston et al., 2014), which is stabilized by salt bridges between the D49 and R52 residues, which are highly conserved in Zur proteins (Fig. S4 A). The RNNNY (R = purine, N = any base and Y = pyrimidine) binding motifs in the target DNA are recognized by Zur monomers. Each Zur_{Ec} dimer docks on the opposite sides of the target DNA (Gilston et al., 2014), and the RNNNYxxxRNNNY element (xxx = a three-base spacer) is the core recognition sequence for one Zur dimer (Fig. S4B). The observation that two Zur dimers bind at overlapping RNNNY motifs led to the identification of the conserved 18 bp dimer-dimer recognition motif **R**xxx**YR**xx**R***Y**xx**YR**xxx**Y** (* indicates the center of the palindrome sequence) (Gilston et al., 2014). Examining the dimer-dimer recognition motif could lead to the identification of possible additional members of the Zur_{Ec} regulon (Gilston et al., 2014). The analysis of the 27 bp duplex DNA (5′-TGTTGATATGTTATTACATTACATATT-3′) from the *atu3184* promoter, which was a protected region that was determined by the DNase I footprinting assay (Fig. 3B), suggested that Zur_{At} may bind to the *atu3184* promoter as a dimer of dimers (Fig. S4C), in accordance with the presence of the ideal **R**xxx**YR**xx**R***Y**xx**YR**xxx**Y** motif 5′-**G**AT A**TG**TT**A***T**TA**CA**TTAC-3′ in the Zur box of *atu3184* (Fig. S4D).

There are similarities between Zur_{At} and $Zur_{Ec,}$ including 1) the conserved coordinating residues for both structural and regulatory Zn-binding sites 1 and 2; 2) the presence of a tyrosine residue corresponding to Y45 of $Zur_{Ec,}$ which was shown to interact with the inner TATA of the Zur box; and 3) the Zur dimer-dimer recognition site (the **R**xxx**YR**xx**R***Y**xx**YR**xxx**Y** motif) was found in the promoter of their target genes. These results suggested that the molecular mechanism of Zur-DNA recognition and Zur repression through the cooperative binding of two Zur dimers to DNA is a likely mechanism in *A. tumefaciens*, similar to *E. coli*.

The general function of Zur in many bacteria is to repress high affinity zinc uptake systems under zinc-sufficient conditions to avoid intracellular zinc overload-mediated toxicity via mismetallation of non-zinc-binding proteins (Foster et al., 2014). Bacteria have evolved a number of specialized zinc-specific adaptations beyond turning on zinc uptake machinery via derepression by Zur to ensure that critical processes continue properly under extreme zinc limitation. Zur also controls these adaptive responses, including zinc sparing and allocation (Capdevila et al., 2016). Zinc sparing involves the increased expression of non-zinc-requiring proteins to replace zinc-containing proteins, such as genes encoding for paralogs of ribosomal proteins, and zinc can be liberated and available for reallocation by zinc chaperones to more essential zinc-dependent proteins for cell growth (Mikhaylina et al., 2018). The *A. tumefaciens atu3184* mutant did not show the EDTA-hypersensitive phenotype. One likely explanation is that the Atu3184 protein may play a minor role and its function could be masked by other redundant proteins that have a similar function. Genes of the Zur regulons from several bacteria also include other regulators, transporters, virulence factors and secreted proteins (Mikhaylina et al., 2018). It is possible that the function of Zur-regulated COG0523 members may be linked with other essential cellular processes in which specialized metal-insertases and/or chaperones are required for highly efficient metal delivery to ensure correct metal cofactor loading under severe zinc-limited conditions. Some Zur-regulated COG0523 members might not function as zinc chaperones but as distributers of other metals. Further study is required to establish the role that the Atu3184 protein plays in the zinc starvation response and to identify the pathway that involves Atu3184.

In conclusion, the *atu3184* gene was identified as a true member of the *A. tumefaciens* Zur regulon based on the following observations: 1) the presence of a Zur box; 2) evidence that Zur directly binds to the promoter region of *atu3184 in vitro*, as indicated by the EMSA and DNase I footprinting analysis; and 3) *in vivo* promoter-*lacZ* reporter assay. The function of *atu3184* encoding a GTPase protein in response to zinc deprivation remains to be elucidated in a future study. The importance of zinc homeostasis at the host-pathogen interface is receiving increasing attention. The competition for zinc availability between pathogens and hosts has implications in determining the outcome of successful infections. Understanding the mechanisms that contribute to zinc homeostasis in pathogenic bacteria could potentially benefit and lead to the development of new therapeutic targets.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.02.008.

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